Temperature-Sensitive Mutations of Bacteriophage T4 Lysozyme Occur at Sites with Low Mobility and Low Solvent Accessibility in the Folded Protein†

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ABSTRACT: Twenty-five different temperature-sensitive point mutations at 20 sites in the lysozyme gene of bacteriophage T4 have been identified. All of the mutations alter amino acid side chains that have lower than average crystallographic thermal factors and reduced solvent accessibility in the folded protein. This suggests that the amino acids with well-defined conformations can form specific intramolecular interactions that make relatively large contributions to the thermal stability of the protein. Residues with high mobility or high solvent accessibility are much less susceptible to destabilizing substitutions, suggesting that, in general, such amino acids contribute less to protein stability. The pattern of the sites of ts substitutions observed in the folded conformation of T4 lysozyme suggests that severe destabilizing mutations that primarily affect the free energy of the unfolded state are rare. These results indicate that proteins can be stabilized by adding new interactions to regions that are rigid or buried in the folded conformation.

The interactions that determine the thermodynamic stability of proteins have been studied extensively, but few general principles have emerged. Studies of mutant proteins with altered stability suggest that the consequences of a mutation depend on the amino acid substitution as well as the structural context in which it occurs (Grütter et al., 1979; Perutz, 1980). For example, the X-ray crystal structures of several temperature-sensitive (ts) and neutral mutants of the lysozyme of bacteriophage T4 have been determined (Grütter et al., 1979, 1987; Alber et al., 1986; Alber & Matthews, 1987). Different mutant proteins contain changes in charge, hydrophobicity, solvent structure, packing, and hydrogen bonding. Simple patterns that characterize stabilizing interactions and quantitatively predict the effects of amino acid substitutions have eluded identification.

In this paper we report the isolation and characterization of an expanded collection of ts mutants of bacteriophage T4 lysozyme. The mutations affect chemically different amino acids, indicating that a variety of noncovalent interactions can contribute to protein stability. In addition, the ts mutations are found to alter only amino acids whose side chains have low crystallographic thermal factors and low solvent accessibility in the X-ray crystal structure of the wild-type protein. This suggests that residues that have well-defined positions and are held relatively rigidly in the folded state make the largest contributions to the stability of the protein.

Materials and Methods

Mutagenesis and Screening. The methods used to generate and screen for ts mutations in phage T4 lysozyme were developed by Streisinger and co-workers (Streisinger et al., 1961, 1966; Okada et al., 1969; 1972; Owen, 1971). Most of the mutations described here were induced by growing T4 *ac q* e^+ for a single cycle in the presence of 1 mg/mL 5-bromouracil or 2-aminopurine (Gray, 1985; Grütter et al., 1987). Temperature-sensitive mutants produced progressively smaller halos at increasing growth temperatures or produced plaques at 43 °C only in the presence of added hen egg white lysozyme (0.2 mg of lysozyme/mL of top agar).

Two ts mutants (DM 841 and DM 407) were identified after treating plasmid DNA containing the T4 lysozyme gene with hydroxylamine in a manner suggested by Volker and Showe (1980).

Temperature-sensitive pseudorevertants of amber mutations at the positions of two tryptophans (residues 138 and 158) were from the collection of George Streisinger [see Inouye et al. (1970)].

Site-Directed Mutants. Several mutants obtained by oligonucleotide-directed mutagenesis (Zoller & Smith, 1984) were found to be ts. Details of these experiments will be published elsewhere. The complete DNA sequence of the noncoding strand of the mutagenized 3′ half of the lysozyme gene was determined (Sanger et al., 1977). The sequenced 3′ gene fragment was used to construct a complete mutant lysozyme gene in the expression vector pHSe5, and a plate enzyme assay was used to screen for reduced lysozyme production at increasing growth temperatures (D. C. Muchmore, unpublished results; Alber & Matthews, 1987).

Cloning and DNA Sequencing. Procedures for cloning and sequencing mutant lysozyme genes from phage T4 have been described in detail (Owen et al., 1983; Grütter et al., 1987; Alber & Matthews, 1987). Briefly, the lysozyme mutation of interest was crossed into a T4 strain (*denA*, *denB*, *56*, and ΔeG19) that produces unmodified DNA in an su⁻ host (*Escherichia coli* BB). This DNA was extracted, digested with *Xho*I, and gel purified. A ~1-kb *Xba–Hin*dIII fragment containing the lysozyme gene was subcloned into M13mp18. The sequence of the noncoding strand of each lysozyme gene was determined by using the dideoxy chain termination method (Sanger et al., 1977). Overlapping sequence information was obtained by using primers that bind at positions 245, 278, 461, and 135.

Calculation of Average Thermal Factor and Solvent Accessibility. The refined X-ray crystal structure of phage T4 lysozyme (Weaver & Matthews, 1987) was subjected to further refinement against a new 1.7-Å resolution data set collected by oscillation photography (K. Wilson, R. Faber, and

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Table I: Temperature-Sensitive Mutants of T4 Lysozyme

mutant[a]	source[b]	isolates[c]
M6I	ts mutant	DM841
L33P	ts mutant	N5, N6, N16, M6
L66P	tight ts mutant	T12
L91P	tight ts mutant	T1
R96H	ts mutant	N20, M22
C97I,V,F	site-directed mutants	
A98V	ts mutant	BU16, BU17, BU18, BU20
A98T	ts mutant	N3
M102T	ts mutant	N19
V103A	ts mutant	M21
Q105G	site-directed mutant	
W126R	ts mutant	N9
W138Y	amber pseudorevertant	R1-75
W138G	site-directed mutant	
R145H	ts mutant	N1, M4
A146V	ts mutant	N22
A146T	ts mutant	N14
V149A	ts mutant	N24
F153C	site-directed mutant	
G156D	ts mutant	BU19, N15, T19
T157I	ts mutant	BU9
W158Y	amber pseudorevertant	MR5
A160T	ts mutant	DM407

[a] The names of the mutant lysozymes are based on the one-letter code for the amino acids. M6I, for example, indicates the replacement of methionine-6 by isoleucine. [b] Tight ts mutants fail to form plaques at 43 °C. Ts mutant plaques make progressively smaller halos after growth at increasing temperatures. [c] Mutants in phage T4 induced with 2-aminopurine are designated with the prefixes M, N, or T. Phage isolates induced with 5-bromouracil have the prefix BU; DM denotes mutants generated in the cloned lysozyme gene by treatment with hydroxylamine.

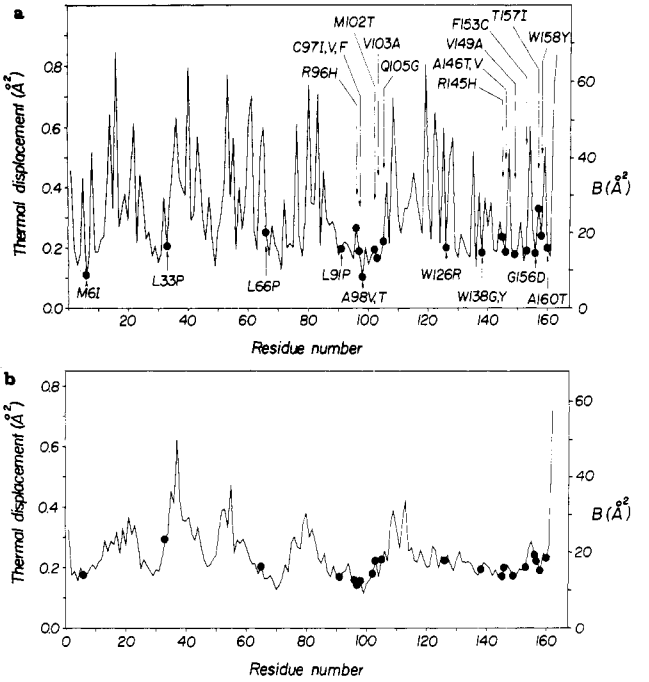

FIGURE 1: (a) Mean square thermal displacement of the side-chain atoms. The displacement for each residue was obtained by averaging the B values of the side-chain atoms (not including the α-carbon). For glycine the value for the α-carbon is plotted. Solid circles indicate the positions of the ts mutations listed in Table I. (b) Mean square thermal displacement of the backbone atoms. The value plotted for each residue was obtained by averaging the crystallographic thermal factor B of the four backbone atoms, CA, C, O, and N.

B. W. Matthews, unpublished results). The TNT package of programs was used for this process (Tronrud et al., 1987). The R value of the current model obtained by using all the data between 6- and 1.7-Å resolution is 16.7%. Root mean square deviations from ideal bond lengths and bond angles are 0.018 Å and 2.26°, respectively. Average thermal factors of each residue were calculated as the simple means of the B values of the main-chain and the side-chain atoms.

The static solvent accessibility A of each residue in the X-ray crystal structure was calculated by the method of Lee and Richards (1971). To obtain suitable reference accessibilities, the molecular graphics program FRODO (Jones, 1982) was used to build a model with the amino acid sequence of phage T4 lysozyme in an extended conformation ($\phi = -139°, \psi = 135°$). The main-chain torsion angles of the three prolines were set at $\phi = -77°$ and $\psi = 146°$, and the side-chain torsion angles χ were set at 180°. The calculated solvent accessibility A_0 of each residue in the extended model was used for comparisons with the X-ray crystal structure.

Results

Using a genetic screen developed by Streisinger et al. (1961, 1966), a collection of independent ts mutations induced in phage T4 lysozyme by the transition mutagens 2-aminopurine and 5-bromouracil has been isolated. The mutant phage produce decreasing amounts of lysozyme activity at increasing growth temperatures. The amino acid substitutions and X-ray crystal structures of five of these ts lysozymes have been reported (Grütter et al., 1979, 1987; Gray, 1985; Alber et al., 1986). Thermodynamic analyses show that these mutant lysozymes are approximately 1–4 kcal/mol less stable than the wild-type protein at 42 °C (Schellman et al., 1981; Hawkes et al., 1984; Becktel & Schellman, 1987).

To characterize additional ts lesions in the collection, the mutant T4 lysozyme genes were cloned into phage M13mp18, and the DNA sequence of the noncoding strand of each clone was determined. The changes in amino acid sequence in these mutants are listed in Table I. Several site-directed mutants with comparable temperature-sensitive phenotypes in the standard plate enzyme assay are also listed. Approximately 30 independently isolated ts mutations have been sequenced, yielding 25 distinct amino acid substitutions. Different randomly induced amino acid replacements at the same site were observed in two cases, namely, threonine and valine substitutions at Ala-98 and at Ala-146.

Most of the amino acid substitutions are located in the C-terminal domain of the molecule [residues 1–11 and 81–164; see Figure 3 of Remington et al. (1978)]. This includes Met-6 → Ile, which is close to the amino terminus in the primary sequence but can be considered as part of the C-terminal domain in the three-dimensional structure of the enzyme. Leu-33 → Pro and Leu-66 → Pro, however, are clearly in the N-terminal lobe (residues 13–66).

To estimate the mobility of each residue in the refined X-ray crystal structure of phage T4 lysozyme, the average thermal displacements were calculated separately for the side-chain (Figure 1a) and the main-chain atoms (Figure 1b). The unidirectional mean square thermal displacement $\langle\mu^2\rangle$ is related to the crystallographic thermal factor B by the equation $B = 8\pi^2\langle\mu^2\rangle$. The B values contain contributions from thermal motion, segmental motion, lattice disorder, and errors in the molecular model. Nevertheless, thermal motion has been found to dominate the observed B values in several crystalline proteins (Petsko & Ringe, 1984).

A striking result is that the ts mutations occur at residues whose side chains have low average mobility. All target side chains have thermal displacements that are less than or equal to the average value ($\bar{B} = 26.5$ Å^2). Most of the sites of ts mutations occur at minima in the plot of side-chain thermal displacements (Figure 1a).

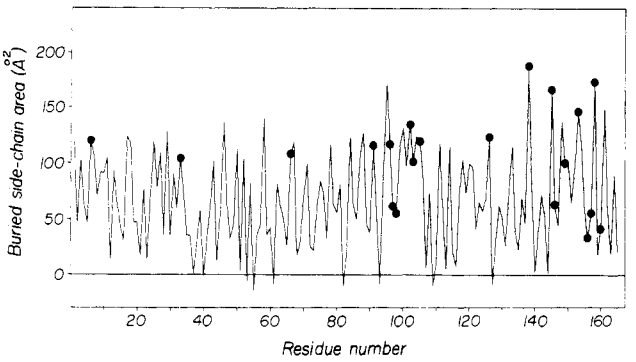

FIGURE 2: Buried surface area for each residue. The ordinate is $A_0 - A$, where A_0 is the side-chain surface area accessible to solvent in a model having the T4 lysozyme sequence in a fully extended conformation and A is the calculated side-chain solvent-accessible surface area in the X-ray crystal structure of phage T4 lysozyme. Dots indicate the ts mutations listed in Table I.

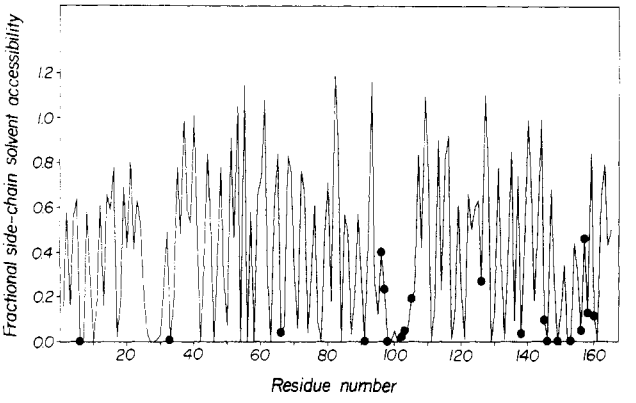

FIGURE 3: Fractional accessible surface area, A/A_0, for the side chains in T4 lysozyme. Dots indicate the positions of ts mutations.

There is also a correlation between the locations of destabilizing mutations and the rigidity of the main chain (Figure 1b). This result may have limited significance, however, because most mutations do not chemically alter the protein backbone. No ts mutations were found in regions of highest backbone mobility. Of the 23 substitutions, only one, Leu-33 → Pro, has a B value higher than the average main-chain thermal displacement ($\bar{B}$ = 20.3 Å^2). The B values of these atoms are, nonetheless, moderate compared to the structure as a whole.

The X-ray crystal structure of T4 lysozyme was also used to calculate the static surface accessibility of each residue. Figure 2 shows the locations of the ts mutations relative to buried side-chain surface area. The side chains with the largest absolute amount of buried surface area are clearly sensitive to destabilizing substitutions. Ts mutations also occur at a number of amino acids was less buried surface area. To place the values for different types of residues on a common scale, fractional solvent accessibilities were calculated (Figure 3).

Figure 3 shows that most of the ts mutations occur at amino acids that are inaccessible or nearly inaccessible to solvent. The ts mutant sites with the highest side-chain fractional accessibility are Thr-157 → Ile (0.49) and Arg-96 → His (0.41). The average fractional accessibility of all the ts sites is 0.13, compared to an average value of 0.40 for the protein as a whole.

DISCUSSION

The 25 ts mutations of phage lysozyme that have been identified (Table I) do not constitute an exhaustive set. If an equal probability of mutation at each base pair is assumed, analysis of the frequencies of the randomly induced mutations in Table I according to the Poisson law indicates that the collection includes 40–60% of the ts sites. This estimate is an upper limit, however, because the observed distribution of ts lesions is dominated by sites with high mutation rates. Nonetheless, this value is high enough to suggest that mutations in a significant fraction of the lysozyme gene have been induced and screened for the ts phenotype.

The amino acid substitutions that destabilize the protein are chemically varied. Hydrogen-binding groups are added by some mutations and removed by others. Net charge is increased, decreased, or left unchanged. Amino acid size and hydrophobic surface area is increased by some mutations and decreased by others. In summary, there is no simple pattern in the nature of the amino acid substitutions that cause temperature sensitivity. Collections of destabilizing mutations in the cro protein of phage λ (Pakula et al., 1986), in staphylococcal nuclease (Shortle & Lin, 1985), in kanamycin nucleotidyltransferase (Matsumura et al., 1986a), and in human hemoglobin (Perutz, 1980; Fermi & Perutz, 1981) show the same trend. These findings are consistent with the view that many different types of noncovalent interactions—including ion pairs, hydrogen bonds van der Waals contacts, and hydrophobic contacts—can make quantitatively comparable contributions to the stability of a protein [e.g., see Brandts (1967) and Matthews et al. (1974)].

Although the observed amino acid changes are chemically varied, most of them cluster in the C-terminal domain of the protein. Since the genetic screen requires that the mutants form active enzyme at low temperature, mutations in the N-terminal domain may have been missed because they produce phenotypes that are either too severe or too mild to be detected. The latter conclusion is supported by several findings. (1) Double frame-shift mutations that cause wholesale changes in 13 of 60 residues in the N-terminal domain nonetheless allow active enzyme to be produced (Terzaghi et al., 1966; Streisinger et al., 1966; Okada et al., 1969, 1972; Streisinger & Owen, 1985). These mutations define relatively insensitive regions where the cumulative effects of multiple substitutions produce only moderate reductions in activity or stability. (2) Substitutions at Cys-54 in the N-terminal domain cause only small changes in stability (Perry & Wetzel, 1986; Alber & Matthews, 1987; T. Alber, S. Cook, W. Becktel, and B. W. Matthews, unpublished results). (3) Side chains in the N-terminal domain are generally more mobile and more exposed to solvent than those in the C-terminal domain (Figures 1 and 3). As a result, the N-terminal domain may provide a proportionally smaller target for destabilizing mutations (see below).

In addition to locating the sites of ts mutations in the tertiary structure, the refined X-ray crystal structure of lysozyme allows the average mobility and relative solvent accessibility of the mutant sites to be examined. Figures 1 and 3 show clearly that the ts mutations alter amino acids that have relatively rigid, well-defined side-chain conformations with low fractional solvent accessibility in the folded protein. If the 20 sites of ts mutations were distributed randomly with respect to the average side-chain B value and solvent accessibility, the probability of finding all 20 sites below the median value is less than 10^{-6}.

Arg-96 and Thr-157 are the most mobile and exposed sites of ts mutations (Figures 1 and 3). The thermodynamic stabilities and X-ray crystal structures of the Arg-96 → His mutant protein and of lysozymes with 13 different site-directed substitutions at residue 157 have been determined (Grütter

et al., 1979; Alber & Matthews, 1987; T. Alber and B. W. Matthews, unpublished results). These studies suggest that the most critical substituents are the γ-hydroxyl group of Thr-157 and the methylene chain and guanidinium nitrogens NE and NH2 of Arg-96. These atoms are among the least mobile or least accessible in their respective side chains (data not shown).

While the locations of temperature-sensitive mutations in T4 lysozyme are highly correlated with low side-chain mobility and low surface accessibility, a complete collection of ts mutants is expected to include apparent exceptions to this pattern. At mobile sites, substitutions that cause extended structural changes (e.g., some X → Pro or Gly → X replacements) could be indirectly destabilizing. A temperature-sensitive phenotype could also arise from mutations that do not appreciably reduce the thermodynamic stability of the protein. For example, some mutations at mobile sites might selectively affect the folding, degradation, or chemical inactivation of the protein in vivo. Anomalously high B values may be associated with locally rigid amino acids in regions undergoing segmental motion or with residues that are incorrectly located in the crystallographic model. These possibilities notwithstanding, such mutant sites are apparently rare in phage T4 lysozyme.

If the target for ts mutations is largely restricted to amino acids with low mobility or low solvent accessibility, the stability of the protein should be relatively insensitive to substitutions at residues that are highly mobile or exposed to solvent. Several types of mutants fulfill this prediction. (1) Lysozymes with low enzyme activity and only marginally reduced thermostability arise from the substitutions Cys-54 → Tyr and Glu-128 → Lys (Grütter & Matthews, 1982; Alber & Matthews, 1987). The side chain of Glu-128 is mobile (B = 46 Å^2) and is exposed to solvent (f = 0.70). Cys-54 is relatively mobile (B = 29 Å^2) but is buried (f = 0) in the wild-type protein (Figures 1 and 3). (2) Ten substitutions of Pro-86 have been constructed by site-directed mutagenesis with little effect on stability (Alber & Matthews, 1987; T. Alber, J. A. Bell, and B. W. Matthews, unpublished results). Pro-86 is largely exposed to solvent (f = 0.62) (Figure 3). The average side-chain thermal factor is 23.8 Å^2, but this modest value is apparently due to the covalent constraints on the pyrrolidine ring. The average side-chain B value for Leu, His, Asp, Arg, Ser, and Cys side chains at position 86 is 42.1 Å^2, well above the average for the protein (26.5 Å^2) (T. Alber, J. A. Bell, and B. W. Matthews, unpublished results). (3) Double frame-shift mutants that produce phenotypes comparable to the point mutants listed in Table I are confined to regions of the protein that have greater than average mobility or fractional accessibility (Figures 1 and 3). These frame-shift mutants affect residues 1–4, 20–22, 22–25, 34–36, 36–40, 73–76, 138–140, and 139–140 (residues 3, 21, 35, and 39 were not changed by the surrounding frame shifts) (Terzaghi et al., 1966; Streisinger et al., 1966; Okada et al., 1969, 1972; Streisinger & Owen, 1985). Since mutations can have cumulative effects on protein stability (Shortle & Meeker, 1986; Matsumura et al., 1986b), many of the amino acid substitutions in these multiple mutants may have little destabilizing effect.

In summary, the target for destabilizing amino acid substitutions appears to be confined to the least mobile and least solvent-accessible residues in phage T4 lysozyme. Mobile or exposed amino acids seem to be more tolerant of mutations. Since crystallographic thermal factors and calculated solvent accessibility are highly correlated, it is difficult to determine if one of these parameters is more important than the other (Westhof et al., 1984; Tainer et al., 1984; Sheriff et al., 1985). Further studies of mutants at the rare buried sites with high B values (e.g., Cys-54) and at exposed sites with low B values (e.g., Val-131) may clarify this issue.

The correlations between protein stability, side-chain rigidity, and low solvent accessibility provide simple rules for using the high-resolution X-ray crystal structure of a protein to predict the sensitivity of each amino acid to destabilizing mutations. In addition, two complementary strategies for stabilizing proteins are suggested. (1) Additional interactions (e.g., hydrogen bonds, ion pairs) that are compatible with the native structure may be engineered in rigid regions of the molecule (Goldenberg, 1985). (2) Mutations that reduce the mobility of nearby interacting groups may be introduced. Such mutations might include replacement of smaller amino acids by compatible larger ones (e.g., Ser → Thr) and substitution of linear side chains by branched or aromatic residues that would fit the native structure.

In addition to providing useful phenomenological correlations, the results reported here may bear on current theories of protein stability. Figure 2 shows that the residues with the most buried surface area are sensitive to destabilizing mutations. This emphasizes the importance of the hydrophobic effect and of van der Waals contacts arising from felicitous packing of the protein interior (Richards, 1977). However, even if the absolute amount of buried surface area is small (Figure 2), residues that are sequestered from solvent in the folded state can be sensitive to destabilizing substitutions (Figure 3). This result is difficult to rationalize on the basis of the hydrophobic effect alone. Instead, it is consistent with the view that groups that become surrounded by protein atoms during folding can form a variety of specific noncovalent interactions that contribute to the difference in free energy between the folded and unfolded states. Groups that are exposed to water in the folded and unfolded states may contribute less to protein stability, because their average environments in the two states are very similar.

The observation that ts mutations alter side chains with low crystallographic thermal factors (Figure 1a) supports the hypothesis that the relative effective concentrations of interacting groups in the folded and unfolded states determines their contribution to protein stability (Creighton, 1983a,b; Goldenberg, 1985). In this view, groups that are constrained by the folded conformation to interact productively can make much stronger noncovalent bonds than groups with greater motional freedom. Substituents whose conformational entropy has been reduced by the protein structure can have a larger fraction of the enthalpy of interaction expressed in the free energy of stabilization. This theory, supported by measurements of the stabilities of intramolecular anhydrides and disulfides (including the disulfides in bovine pancreatic trypsin inhibitor), predicts that the most rigid parts of folded proteins make the largest contribution to stability (Creighton, 1983a,b). The correlation shown in Figure 1a is consistent with this prediction.

In closing, it should be noted that mutations in general, including those shown in Table I, can alter the free energies of both the folded and unfolded states of the protein. In principle, mutations that predominantly affect the free energy of the unfolded state could occur anywhere in the protein. Their locations would not be expected to correlate with specific properties of the folded form. Since the observed ts mutations in phage T4 lysozyme occur primarily at sites with low mobility or low solvent accessibility in the X-ray crystal structure, it is tempting to conclude that the observed reductions in thermodynamic stability generally involve significant effects on

the folded conformation. The free energy of the unfolded state may be less sensitive to point mutations. These properties may reflect a greater ability of the unfolded state to compensate for amino acid substitutions. The validity of these conclusions, however, cannot be evaluated without more direct thermodynamic and structural studies of the folded and unfolded states of mutant proteins.

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